Effects on Murine Epidermal Langerhans Cells of Drugs Known to Cause Recrudescent Herpes Simplex Virus Infection in a Mouse Model

Neil A. Williams and Terry J. Hill
Department of Pathology and Microbiology, School of Medical Sciences, Bristol, U.K.

A number of agents have been shown to alter the latent state of herpes simplex virus in murine sensory ganglia. However, it seems that effective triggers of recrudescent disease must act not only to reactivate latent HSV infection, but also to create a favorable environment in the skin for viral replication. The possibility that alteration of the local Langerhans cell population is one way in which effective triggers of recrudescence may act has been investigated. Of the agents tested, which affect latent HSV, only DMSO significantly altered the numbers of ATPase-bearing Langerhans cells in the epidermis, maximally reducing their density by 83% in 48 h. Xylene and retinoic acid had no discernible effect on numbers of ATPase-staining cells over the 4 d tested. However, the extent to which agents reduced ATPase-staining cell numbers did not correlate with their ability to affect the antigen-presenting capacity of the cells in HSV-specific T-cell proliferative assays in vitro. Xylene and retinoic acid markedly reduced the accessory cell function of epidermal cell suspensions, whereas DMSO had no effect. *J Invest Dermatol 97:933–937, 1991*

Notable features of the herpes viruses are their ability to escape complete clearance by the host immune system and their ability to establish a latent infection that may persist for the life of the animal. In the case of herpes simplex virus (HSV), latent infection is established in neuron cell bodies of the sensory ganglia [1,2]. In some individuals the virus is periodically reactivated and spreads down the nerves to the epidermis where either recurrence (asymptomatic) or recrudescence (symptomatic) may occur (see [3] for detailed description of terminology). The factors that control whether recurrence or recrudescence follow reactivation remain unclear. Individuals suffering from recrudescent disease do not appear to be overtly immunosuppressed, as problems with other pathogens are rarely apparent [4]. Furthermore, the infection is usually cleared quickly and occurs in the face of demonstrable humoral and cellular immune reactivity [3]. Therefore, in order to understand the immune mechanisms involved it is necessary to study the factors associated with the induction of the local immune response to the virus.

Langerhans cells (LC) are considered to be central to the initiation of immune reactivity to epidermally encountered antigens such as HSV. They can take up a variety of immunogens in vivo [5–7], and constitutively express class II MHC antigens [8]. In addition, they are capable of presenting soluble and particulate antigens, including HSV, to primed T cells in vitro [9–13]. Following secondary challenge with contact sensitizers in vivo there is a rapid migration of LC bearing antigen to the lymph nodes, and at the same time apposition of LC to lymphocytes in the epidermis itself is observed [14,15]. These observations, along with the in vitro data, strongly suggest that LC may present antigen locally in the delayed-type hypersensitivity reaction.

These observations prompt the obvious suggestion that factors such as stress, UV light, and skin trauma, which are known to cause recrudescence in man, may act, at least in part, by inhibiting LC function. Indeed, UV light has been shown to reduce numbers of LC in the epidermis (as defined by staining for ATPase, and class II MHC antigens [16]), and inhibits their function as presenting cells in vitro [17]. However, attributing the alteration in HSV infection to the effects of such agents on LC has proved difficult due to the multiple actions that these and other experimental stimuli have on the infected animal. For instance, UV light, even at sub-erythemal doses, causes pathologic changes in the skin [18]. Furthermore, employing different wavelengths of UV light allowed Noonan et al to establish that immunologic unresponsiveness to contact sensitizers was maximally induced at a wavelength distinct from that which caused an apparent depletion of LC [19].

A further factor that has inhibited such studies has been the lack of satisfactory models of recrudescent HSV infection. However, work from our laboratory has established a model with which to study recrudescent infection of the mouse pinna with HSV-1 [20]. Using this system, we have shown that, in addition to UV light and stripping the skin with cellophane tape, a number of chemicals can induce reactivation, recurrence, and in some cases recrudescent HSV infection [21,22]. Interestingly, all three chemicals tested (xy-

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Reprint requests to: Neil A. Williams, Department of Microbiology and Pathology, University of Bristol, The Medical School, University Walk, Bristol, B58 1TD U.K.

Abbreviations:
ADP: adenosine diphosphate
APC: antigen presenting cell
ATP: adenosine tri-phosphate
CPM: counts per minute
DMSO: dimethyl sulphoxide
EC: epidermal cell
EDTA: ethylenediamine tetra-acetic acid
HSV: herpes simplex virus
MHC: major histocompatibility complex
LC: Langerhans cell
PBS: phosphate-buffered saline
pfu: plaque-forming unit
PG: prostaglandin
UV: ultraviolet

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lene, retinoic acid, and dimethyl sulphoxide [DMSO]) acted as "ganglion triggers" [21] (cause reactivation of latent virus) but only xylene and retinoic acid induced significant recrudescent disease. From this it was suggested that the necessary "skin trigger" [21] is not provided by some stimuli such as DMSO. Because the direct effects of the drugs on latent virus have been established, the mouse model provides an excellent opportunity to look for a correlation between their effectiveness as "skin triggers" and their impact on LC.

MATERIALS AND METHODS

Animals The mice used were 6-week-old female inbred NIH mice bred in the departmental animal facility from stock originally purchased from Olac 1976 Ltd.

Virus A strain of HSV-1, strain SCI6, originally isolated from a labial lesion at the PHLS, Bristol, U.K. [20] was used. Stock virus for use in vitro was prepared in Vero cells and inactivated by exposure to a lethal dose of UV light before use. The titre of virus and the efficacy of the inactivation procedure was routinely tested by plaque assay [20]. Virus stocks for immunization of animals were prepared in Hep-2 cells. The use of different cell lines for the preparation of virus to immunize mice and to add to in vitro cultures of lymphocytes from those animals avoided the possibility that the responses in the cultures could be directed in part at cellular components contaminating the virus preparation.

Immunization Mice were immunized sub-cutaneously in the flank with 1×10^5 plaque-forming units (pfu) of live virus 10 d prior to initiation of lymphocyte cultures.

Demonstration of ATPase-Bearing Cells in Epidermal Sheets Split pinnae were incubated in 4 mM EDTA (tetra-sodium salt, Sigma Chemical Company, Poole, U.K.) in PBS for 2 h at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The epidermis was removed and washed by flotation on several changes of PBS. Tissue was fixed and stained for ATPase activity according to the method of Juhlin and Shelley [23] but using the modification suggested by Chaker, Tharp, and Bergstresser [24], whereby ADP (sodium salt from equine muscle, Sigma grade IV) rather than ATP was used as a substrate.

Stained epidermal sheets were mounted onto slides in Apathies mounting medium (Raymond A. Lamb, U.K.). The numbers of ATPase-bearing cells/mm² for each epidermal sheet was calculated as an average derived from counting five fields of view at a magnification of ×320.

Cell Preparation Spleens were minced through stainless steel mesh into phosphate-buffered saline (PBS). The cell suspension was decanted and washed 3 times with PBS by centrifugation at 1000 rpm. T cells were purified by passage of spleen cell suspensions through rabbit anti-mouse immunoglobulin (Ig)-mouse Ig-coated glass bead affinity columns [25]. The cells collected were greater than 95% T cells as defined by immunofluorescent staining with anti-Thy 1 antiserum assessed by flow cytometry with an EPICS (Coulter) (data not shown).

Epidermal cells (EC) were dissociated from the pinnae of mice as previously described [26]. In brief, split pinnae were incubated in 4 mM EDTA (tetra-sodium salt, Sigma Chemical Company, Poole, U.K.) in PBS for 2 h at 37°C in a humidified environment of 5% CO_2 and 95% air. The epidermis was removed from the dermis and the epidermal sheets were floated on 1% trypsin (Difco, Detroit, MI) in PBS for 75 min at 37°C. The resulting cell suspension was washed at least 3 times by centrifugation in PBS and treated with 1500 rads from a cesium source (Gravatom Industries Ltd., Gosport, U.K.) prior to use in culture. The viability of all the cell suspensions was assessed by their ability to exclude the dye trypan blue. Cell preparations with viability lower than 90% were discarded.

T-Cell Proliferative Cultures The culture medium was the alpha-modification of Eagle's minimal essential medium (Flow Laboratories, Irvine, Scotland; Gibco Europe Ltd., Paisley, Scotland) supplemented with 4 mM L-glutamine (Gibco), 5×10^{-5} M 2-mercaptoethanol (Sigma), 100 U/ml benzyl penicillin (Glaxo Ltd., Greenford, U.K.), 20 mM Hepes (Sigma), and 0.5% fresh autologous normal mouse serum. Therefore, no heterologous serum additives were present in the cultures.

Cultures contained 1.25×10^6 immune T-cells/ml in 0.2-ml volumes in 96-well microtiter plates (Sterilin Ltd., Feltham, UK). EC were used at a predetermined optimal concentration of 2.5×10^5 cells/ml [12,13]. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After an appropriate period in culture, three replicate 0.2-ml microtiter cultures were pulsed with 1 μCi per well of ^{3}H-thymidine (73 to 85 Ci/mM; NEN Research Products, Boston, MA) in 20 μl of medium. Following 6 h of further culture, the samples were harvested onto glass fiber filter mats (Whatman Labsales Ltd., Maidstone, U.K.), and the ^{3}H-thymidine incorporated into newly synthesized DNA was measured by conventional liquid scintillation procedures in a LKB rackbeta (LKB-Wallac, Turku, Finland). Results are expressed as the mean counts per minute (cpm) with the standard error of the mean of the replicate cultures. Statistical significance was evaluated by Student t test.

Treatment of Mice with Chemical Agents Fifty μl of DMSO (100%), xylene (50% in ethanol), or retinoic acid (0.01% in acetone) were applied to the surfaces of the pinna [22].

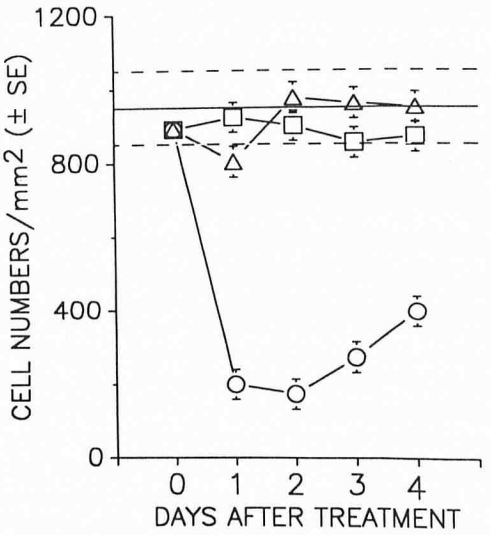

Figure 1. Effects of drugs on numbers of ATPase-bearing cells/mm² of epidermis. Mean numbers of ATPase-staining cells/mm² in epidermal sheets from the upper surface of the right pinnae of 4-week-old mice. Figures represent the mean of five fields of view from each of five animals per group per timepoint. The right pinnae of groups of mice were treated with DMSO (○), xylene (△), or retinoic acid (□). The mean number of cells in pooled control epidermal sheets (n = 20) (——) ± SD (---) is also shown.

RESULTS

Alterations in Numbers of ATPase-Bearing Langerhans Cells The numbers/mm² of ATPase-bearing cells in the epidermis of the pinna was assessed daily for 4 d following treatment of the ear with xylene, retinoic acid, or DMSO (Fig 1). None of the chemicals caused a change in the numbers of staining cells in the untreated left pinna, and therefore cell counts from these samples were pooled with those from the untreated control animals to give a more accurate control range. A highly significant reduction in numbers of ATPase-bearing cells followed the topical application of DMSO. Numbers of staining cells were reduced by 77% 1 d after treatment, and by 83% after 2 d. Thereafter, the numbers recovered though they remained significantly lowered after 4 d. A separate experiment (data not shown) revealed that numbers of ATPase-

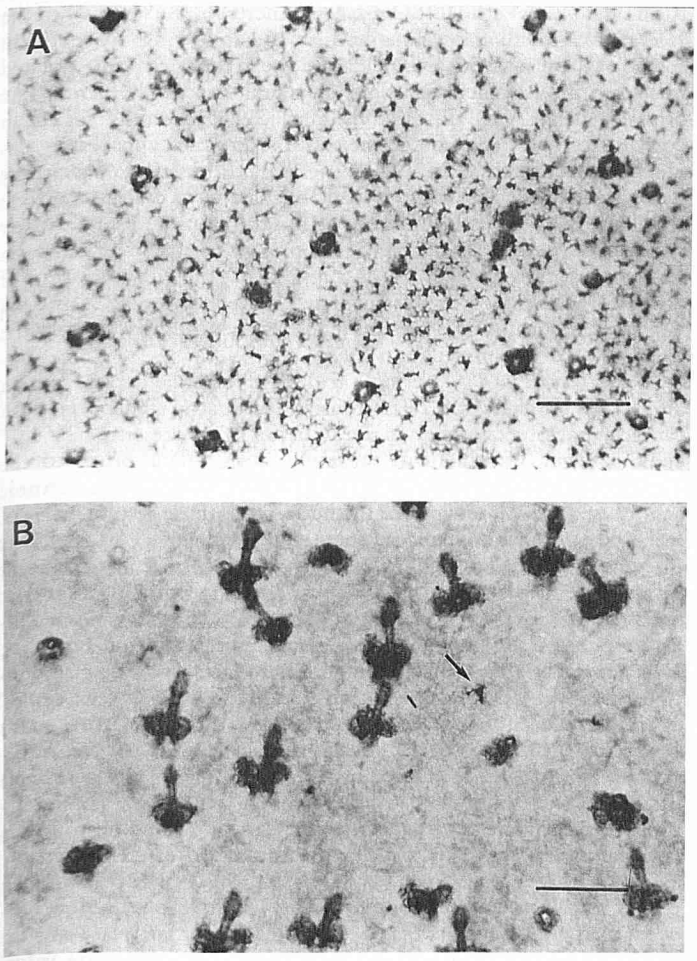

Figure 2. Depletion of ATPase-bearing cells in the epidermis by DMSO. ATPase-stained Langerhans cells on whole epidermal sheets from the right pinna of *(A)* untreated mouse and *(B)* mouse treated on the right pinna with DMSO 24 h previously (*arrow* indicates one of the few remaining stained cells). *Bar,* 50 μm.

positive cells recovered to control levels on day 7 after application of DMSO. The dramatic nature of the reduction in ATPase-staining cell numbers following application of DMSO is further illustrated in Fig 2.

Densities of ATPase-positive cells remained well within the range of untreated control values following the topical application of either xylene or retinoic acid (Fig 1).

Characterization of the T-Cell Proliferative Response to HSV-1 T cells from HSV-immune mice were cultured in the presence of unselected spleen cells (as a source of antigen-presenting cells) and a variety of concentrations of UV-inactivated HSV-1. Proliferation in the cultures was measured over a timecourse and the peak response, which occurred after 4 d, is shown in Fig 3. T-cell division occurred in the presence of a range of virus concentrations; however, the reaction was maximal with 1.2×10^7 erstwhile pfu/ml. The response of the T cells was specific for viral components as proliferation to an equivalent concentration of mock virus was not significantly above the background. Further, Fig 3 shows that the T-cell response to HSV is dependent on the addition of a source of APC. Thus, proliferation of the T cells in the absence of added unselected spleen cells was not significant above background.

Effects of Chemicals on the Ability of Epidermal Cells to Present HSV to Immune T Cells We have previously established that suspensions of epidermal cells can substitute for spleen cells as a source of APCs in our T-cell cultures [12,13]. Further, it is the I-A–bearing Langerhans cells in the epidermal cell suspensions that present the virus to T cells [13]. Therefore, experiments were set up to investigate the effects of topical application of xylene, retinoic acid, or DMSO on the ability of LC subsequently derived from the pinna to present HSV to T cells (Fig 4). In all three experiments, provided virus was also present, strong proliferative responses were stimulated by epidermal cell suspensions from normal non-immune mice. Cells isolated from the pinna 24 h after the local administration of DMSO did not differ in their ability to present HSV to T cells from those derived from untreated mice (Fig 4*A*). In contrast, epidermal cells from pinna that had been treated with xylene or retinoic acid showed a marked decrease in their ability to stimulate proliferation against the virus (Fig 4*B*, *C*). In the case of xylene, the response was not significantly above the small reaction that occurred in the absence of APC (Fig 4*B*). T-cell division in the presence of epidermal cells derived from retinoic acid-treated animals was significant ($p < 0.01$). However, cells from treated animals produced a 57% lower reaction than those from untreated mice, a significant difference ($p < 0.001$) (Fig 4*C*).

DISCUSSION

Although the chemicals we have studied have long been known to cause inflammation when applied to the skin [22], the present investigation is the first report of their effects on cutaneous LC. Over the period of 4 d tested, topical application of xylene or retinoic acid to the pinna of mice did not significantly affect the numbers of cells staining for ATPase in the skin. In marked contrast, treatment with DMSO caused a dramatic depletion of ATPase-stained cells in the epidermis of the pinna; the reduction of staining cell numbers reached a maximum of 83% 48 h after application of the DMSO. Caution is, however, necessary in the interpretation of this result

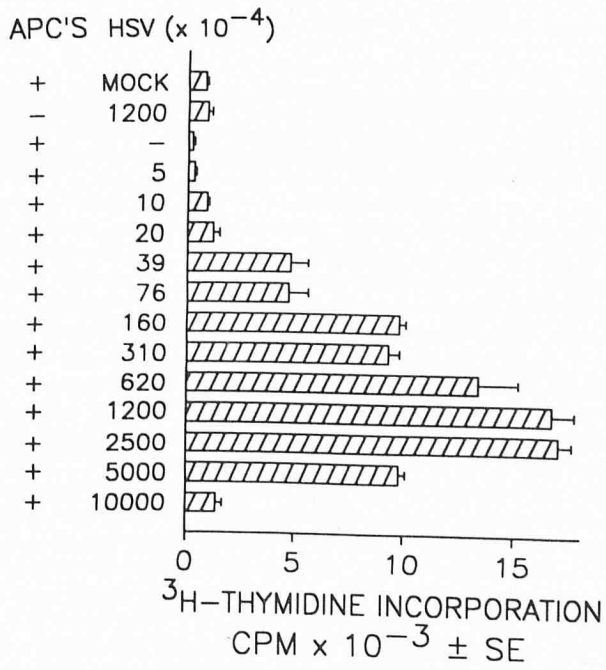

Figure 3. T-cell proliferative response to HSV. Peak proliferative response (day 4 of culture) of splenic T cells from HSV-immune 6-week-old mice in the presence or absence (as indicated) of a 1:1 ratio of gamma-irradiated syngeneic spleen cells as antigen-presenting cells. Cultures contained either no antigen, HSV (at the range of concentrations indicated), or a mock virus preparation at a dose equivalent to 1×10^7 erstwhile pfu/ml.

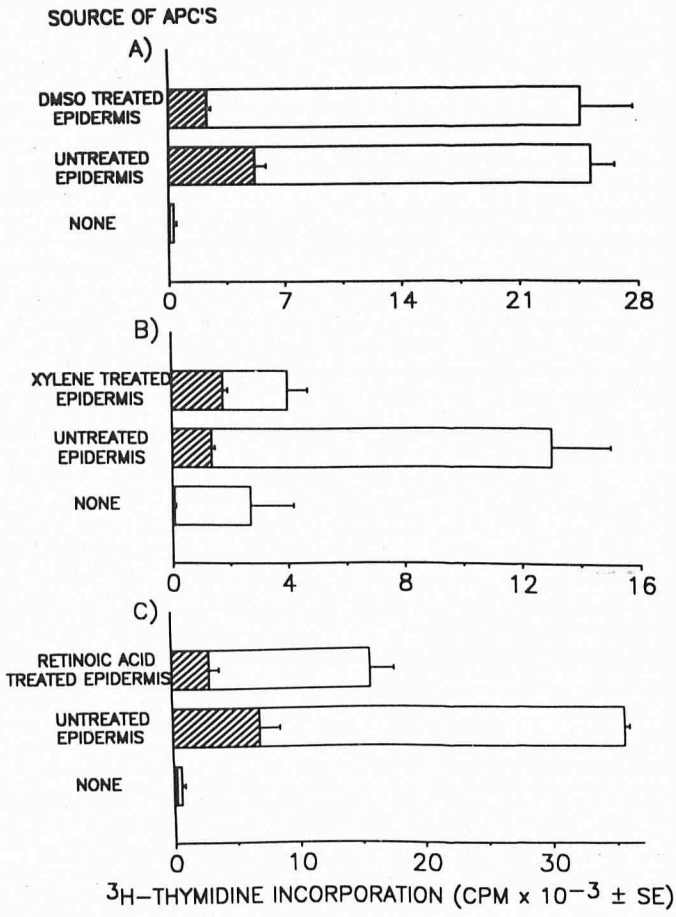

Figure 4. Effects of drugs on the capacity of epidermal cells to present HSV to immune T cells. The capacity of gamma-irradiated epidermal cells from the pinnae of 6-week-old mice, treated 24 h previously on the pinnae with *(A)* DMSO, *(B)* xylene, and *(C)* retinoic acid, to present HSV to immune T cells was compared with that of epidermal cells from untreated mice. The peak proliferative responses (day 5 of culture) in the absence of antigen (▨), and in the presence of 1.2×10^7 erstwhile pfu/ml HSV (□) are shown.

because electron microscopic examination of epidermis from mice treated with DMSO revealed that cells with Birbeck granules (characteristic of LC) remained in the epidermis (although in many cases such cells were vacuolated and had retracted membranes; unpublished results). In this respect, the effect of DMSO is similar to that of UV light where the apparent disappearance of LC from the skin has been shown to be due to their loss of staining for surface ATPase and for Ia molecules [27]. Although DMSO is known to be rapidly absorbed through the skin, its effects on LC were apparently not systemic, as the numbers of ATPase-staining cells in the untreated pinna were not altered.

Despite the morphologic changes and the loss of ATPase staining, cells from the epidermis of mice treated with DMSO were able to present HSV antigens to immune T cells as efficiently as cells derived from untreated controls. Because it has been previously established that it is the LC that present such antigens in our culture system [12,13], as well as those of others [9–12], this result suggests that there is no lack of functionally active LC in the epidermis of DMSO treated mice. The possibility does exist that, following treatment with DMSO, keratinocytes, which are also in our cultures, are induced to express class II MHC antigens and that the keratinocytes therefore become able to present antigen. However, the implication is that following application of DMSO to the skin the epidermis remains capable of inducing a T-cell response to HSV. In other studies, we have shown that similar treatment of the skin of the mouse pinna with DMSO markedly increased the severity of the HSV infection that develops at that site following zosteriform spread of the virus from an inoculation site in the neck [28]. Treated mice also showed increased signs of neural infection. Hence, like other agents (UVB [18], prednisolone [29], hypertonic saline in conjunction with abrasion of the skin [30]), which apparently deplete LC in the skin, DMSO also increases the severity of primary herpetic infection. However, all such agents have multiple effects that may alter the replication of virus in the skin or at some other site. Indeed, in the case of DMSO, our present results, together with those of our previous study [28], suggest that the enhancement of zosteriform infection is not mediated via effects on LC but via enhanced replication of virus in the sensory neurons that supply the dermatome of the inoculation site.

In contrast to DMSO, retinoic acid and xylene, neither of which altered the numbers of ATPase-bearing cells in the epidermis, both caused a dramatic reduction in the ability of LC to present HSV to T cells in vitro. Treatment of the pinna with xylene prior to the isolation of the EC totally abrogated the response, whereas retinoic acid caused a 57% reduction. In the light of these results, it is of interest to compare the effectiveness of these chemicals as inducers of reactivation of latent HSV (in the ganglion) and recrudescent skin disease in the mouse ear model [22]. From this it is clear that depression of antigen-presenting activity of LC correlates with ability to induce recrudescence (xylene and retinoic acid). In contrast, DMSO, although an effective inducer of reactivation in the ganglion, is a poor inducer of recurrent disease and has no effect on LC as antigen-presenting cells. It is notable that this correlation exists only from the functional data in this study. It appears that a reduction of LC numbers as assessed by ATPase staining is not a reliable indicator of the immune competence of the epidermis. The functional results provide evidence that at least one component of an effective "skin trigger" [21] may be inhibition of LC function. Whether the chemicals act directly on the LC or mediate their effects via activity on other components of the skin is unclear. Indeed, many other factors may be involved, e.g., hyperplasia of the local keratinocytes making them more susceptible to virus infection. However, all three chemicals cause some degree of inflammation [22] and it is likely that the degree of hyperplasia would be similar. If the chemicals are influencing local immune responses, it is possible that their effects are mediated via one or more of the inflammatory agonists that they are known to induce in the skin [22]. Histamine, 5-HT, and substance P are released rapidly following application of the chemicals but their presence is transient and, in the case of recurrent infection, they would probably have disappeared before virus enters the skin. However, it is noteworthy that following treatment of the skin with retinoic acid or xylene, prostaglandin E levels were raised for several days in the epidermis but were depressed following DMSO treatment [22]. PGE has a number of effects including enhancement of HSV replication in culture [31] and depression of accessory cell activity by peritoneal macrophages [32]. Hence, PGE may have the potential to act as a "skin trigger" both directly by increasing the susceptibility of skin cells to infection and indirectly by mediating local immunosuppression via depression of LC function.

In conclusion, our work has shown that in studies of factors that affect LC it is important to include an assessment of their immunologic function. Furthermore, it appears that the ability of agents to act as inducers of recrudescent herpetic skin disease in the mouse is linked to their ability to depress LC function. The mechanism by which such depression is mediated, as well as other factors involved, requires further investigation.

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